AUTORADIOGRAPHIC STUDY OF THE DEVELOPING CHICK EMBRYO ESOPHAGUS[1]

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ABSTRACT

Autoradiography was employed to examine the incorporation pattern of ^{3}H-thymidine and ^{3}H-uridine into presumptive stratified squamous epithelial cells and prospective degenerating cells of the chick embryo esophagus. The incorporation pattern of ^{3}H-thymidine suggests that esophageal epithelial nuclei in the vesiculated region undergo interkinetic migration, synthesizing DNA at the basement membrane and moving to the free surface of the vesicles to complete the mitotic phase of the cell cycle. Incorporation patterns in prospective degenerating cells show a cessation of DNA synthesis correlating with the posterior-to-anterior degeneration gradient. No regionally specific pattern of ^{3}H-uridine incorporation was observed.

INTRODUCTION

The description of esophageal organogenesis in *Lillie's Development of the Chick* (Hamilton, 1952) has been modified by Allenspach and Hamilton (1962). The esophagus occludes at 5 days of development by fusion of the roof and floor, and reopens by a process of vesiculation at 7½–8 days. In a subsequent investigation, using the mitotic inhibitor colchicine, Allenspach (1964) observed few mitotic figures in the occluded region, whereas numerous mitotic figures appeared around vesicles during the period prior to reopening, indicating that cell proliferation probably is not a major factor in the occlusion process. The accumulation of mitotic figures adjacent to the vesicles indicated an increase in cell population that is responsible for the increase in tissue volume during elongation of the neck.

The interpretation that degeneration of central epithelial cells between numerous vesicles effects the reopening of the occluded esophagus in a posterior-to-anterior gradient (Allenspach and Hamilton, 1962) was later confirmed in a study on the crooked-neck dwarf mutant chick embryo (Allenspach, 1966). Extensive cell degeneration is not observed during esophageal organogenesis in the crooked-neck dwarf embryo and, as a result, the organ fails to reopen. The result is a highly vesiculated esophagus with numerous epithelial bridges binding the roof and floor in homozygous mutant embryos 8 days and older (Allenspach, 1966). These findings support the hypothesis that cell death is essential to the reopening process.

The cytochemical localization of RNA during esophageal organization indicated intense basophilia in epithelial cells lateral to primary vesicles and in necrotic nuclei (Allenspach and Hamilton, 1962). However, no relationship between basophilia and cell degeneration was made.

The purposes of this study were to identify the origin of the proliferative cell population in the embryonic esophagus and to determine the relationship of DNA and RNA synthesis to differentiation of esophageal epithelium using tritiated precursors.

MATERIALS AND METHODS

Fertile White Leghorn eggs, obtained from a local hatchery, were incubated at 38°C for 5 to 9 days. Prior to treatment of embryos with radioactive precursors, the egg shells were sterilized with a solution of iodine in 95% ethyl alcohol, and a window was made in the shell with a file. The chorio-allantoic membrane was dropped and a measured amount of the appropriate radioactive precursor was

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pipetted onto the area vasculosa (Langman and Nelson, 1968). Embryos of selected stages received 0.2 ml of sterile chick Ringer's saline solution containing either 20 μCi of ^{3}H-thymidine (S. A., 6.7 Ci/mM) or 20 μCi of ^{3}H-uridine (S. A., 2 Ci/mM), both obtained from New England Nuclear Corporation. Following administration of the experimental solution, the window was sealed with cellophane tape and the egg was returned to the incubator. Control embryos were treated with 0.2 ml sterile chick Ringer's saline solution and processed in the same manner as radioactively treated embryos.

At each stage, embryos treated with ^{3}H-thymidine were removed from the shell at ½, 1, 2, 4, and 8 hours after initial labeling. The length of exposure to ^{3}H-uridine was 2, 4, and 12 hours for each stage examined. All embryos, treated and control, were fixed in Carnoy's fixative (1 part glacial acetic acid, 6 parts absolute ethyl alcohol, 3 parts chloroform), the necks isolated, and the embryos staged according to the criteria of Hamburger and Hamilton (1951).

Following dehydration in alcohol and clearing in xylene, the necks were embedded in 53–55° Histowax (Coleman, Matheson, and Bell). Serial sections of neck tissue, 7μ thick, were placed on slides "subbed" with a 1% gelatin solution (Gude, 1968). The sections were coated with diluted Ilford K-5 liquid emulsion (1 part emulsion: 6 parts distilled water), to which 5 drops of 0.5% aqueous sodium

EXPLANATION OF PLATE 1

All autoradiographs represent sections of embryonic esophagus from chick embryos treated with 20 μCi of either ^{3}H-thymidine or ^{3}H-uridine. The labeled precursor and the period of incorporation are indicated in the captions. Sections were stained with Harris' hematoxylin following development of the emulsion. The scale-line on each photomicrograph represents 30μ.

FIGURE 1. Autoradiograph of the occluded region (stage 28–29) following a 4-hour treatment with ^{3}H-thymidine. Intense label is localized mainly in basal nuclei. Lightly labeled areas indicate future locations of the primary bilateral vesicles. ×420.

FIGURE 2. Autoradiograph of the anterior occluded region (stage 32) indicating uptake of labeled thymidine following an 8-hour exposure. Virtually all label is located in cells at the basement membrane. The lightly labeled internal epithelium represents the future site of a primary vesicle. ×440.

FIGURE 3. Cross section through the vesiculated region (stage 28–29) showing the incorporation pattern of labeled thymidine after 1 hour of exposure. The nuclei at the basement membrane are heavily labeled, with little label adjacent to the vesicles. Metaphase figures, indicated by arrows, are consistently unlabeled following 1 hour of exposure to ^{3}H-thymidine. The central area between the bilateral vesicles is almost devoid of labeled nuclei. ×720.

FIGURE 4. Cross section through the multivesiculated region (stage 28–29) after treatment with ^{3}H-thymidine for 4 hours. There is intense label in cells peripheral to the vesicles, with a lack of incorporation in the epithelial bridges separating the vesicles. ×440.

FIGURE 5. Cross section through the vesiculated region showing the incorporation pattern of labeled thymidine after 8 hours of treatment. Nuclei in the epithelial bridges show some incorporation of radioactive label. ×440.

FIGURES 6 and 7 are representative cross sections of the vesiculated region of the esophagus in a stage 35 chick embryo showing the incorporation of ^{3}H-thymidine at anterior and posterior levels, respectively, following an 8-hour exposure period. The epithelial cells constituting the epithelial bridges between the numerous vesicles are prospective degenerating cells, that is, they are destined to degenerate during the reopening process. The first indications of cell degeneration and reopening are observed at posterior levels of the vesiculated region.

FIGURE 6. The dense spherical bodies in the epithelial bridges represent nuclei of epithelial cells with overlying accumulations of silver grains indicating incorporation of ^{3}H-thymidine. Densely labeled nuclei are also evident in epithelial cells at the basement membrane. ×190.

FIGURE 7. The accumulation of silver grains over nuclei is restricted primarily to epithelial cells located near the basement membrane. Note the paucity of labeled nuclei in the epithelial bridges. ×190.

FIGURE 8. Cross section through the anterior vesiculated region (stage 31) following a 2-hour exposure to ^{3}H-uridine. The basal nuclei lateral to the vesicles are intensely labeled. ×460.

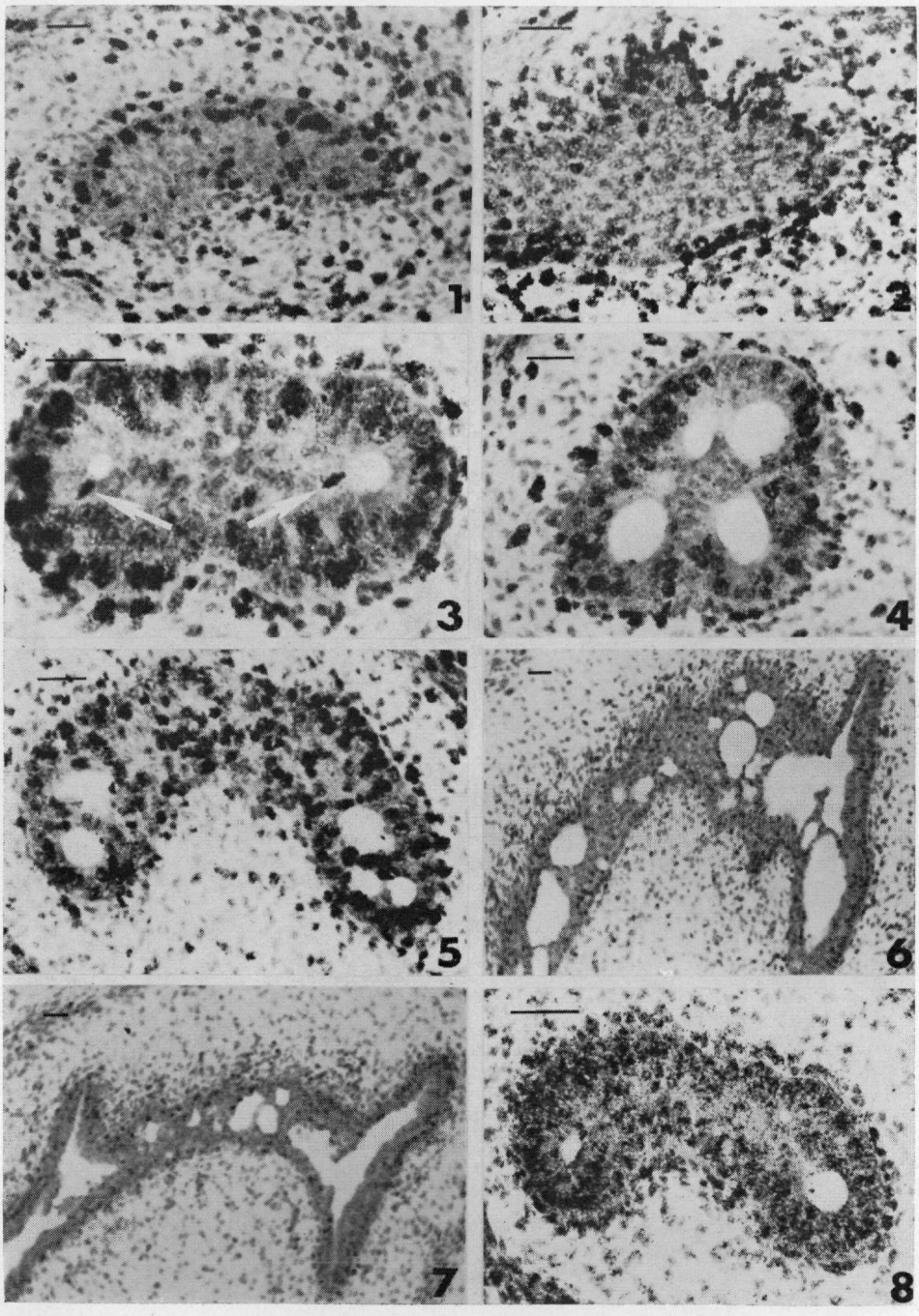

PLATE 1

lauryl sulfate had been added. The emulsion was dried in a light-proof forced-air drying box for 2 days at room temperature, after which time the slides were transferred to light-proof slide boxes containing Drierite and stored at 4°C for the remainder of the exposure period. Suitable autoradiographs of ^{3}H-thymidine-labeled material were obtained after a total of 8 days under emulsion; ^{3}H-uridine-labeled material was developed after a total of 10 days under emulsion. All sections were developed in Kodak D–19 following the method of Weston (1967). Sections were subsequently stained with Harris' hematoxylin, dehydrated, cleared, and mounted.

RESULTS

Special emphasis in this study was placed on the pattern of incorporation of ^{3}H-thymidine and ^{3}H-uridine into cells of the occluded and vesiculated regions of the developing esophagus. The pattern of incorporation of tritiated precursors into esophageal epithelial cells of the posterior open region was similar to the pattern observed in the vesiculated region. Esophageal cells of embryos treated with 20 μCi of ^{3}H-thymidine showed good incorporation, and the embryos suffered no apparent ill effects during treatment. Anomalous pycnotic cells, which could occur upon overdose of ^{3}H-thymidine (Weston, 1967), were not observed in the treated embryos.

The same basic pattern of incorporation of ^{3}H-thymidine into esophageal epithelial cells was observed in the occluded region throughout all stages and at all treatment times. Short-term exposure of embryos to ^{3}H-thymidine ($\frac{1}{2}$, 1, 2 hours) showed numerous silver grains over nuclei at the basement membrane. After four hours of exposure to ^{3}H-thymidine, silver grains also became evident over epithelial nuclei in the medial portion of the occluded esophagus (fig. 1). Following 8 hours of treatment, nuclei with superimposed silver grains were still evident in the medial portion of the esophagus, but intense accumulations of silver grains had obscured the nuclei located at the basement membrane (fig. 2).

Similarly, within the vesiculated region, nuclei situated near the basement membrane were blackened by the large number of overlying silver grains following 1 hour of exposure to ^{3}H-thymidine (fig. 3). However, chromosomes of mitotic figures and nuclei positioned adjacent to vesicles both failed to show incorporation, as evidenced by the lack of associated silver grains (fig. 3). The two mitotic figures in Figure 3 (arrows) contained no silver grains over the chromosomes. As duration of the treatment was increased to 4 hours, silver grains appeared over nuclei and chromosomes of mitotic figures adjacent to the vesicles in the vesiculated region (fig. 4). Although some medially disposed nuclei contained silver grains following 4 hours of exposure to ^{3}H-thymidine, fewer cells in this location contained label than was the case at the basement membrane. Nuclei of epithelial cells between the vesicles in esophagi through stage 33 ($7\frac{1}{2}$–8 days) accumulated large numbers of silver grains following an 8-hour exposure to ^{3}H-thymidine. Within the same time frame, heavy concentrations of silver grains were observed to accumulate over nuclei situated at the basement membrane and over mitotic figures and nuclei located adjacent to the vesicles (fig. 5).

Moving posteriorly, a serial study of the developing esophagus of stage 34 and 35 embryos (8 and 9 days) revealed that nuclei of prospective degenerating cells (*i.e.*, cells occupying positions between the bilateral vesicles) in the anterior vesiculated regions contained numerous silver grains (fig. 6). However, nuclei of prospective degenerating cells in similar relative positions at most posterior levels were void of silver grains (fig. 7). The greatest difference in relative numbers of grains over labeled nuclei was recognized on the extreme anterior and posterior ends of the vesiculated region. Following an 8-hour exposure of chick embryos to ^{3}H-thymidine, nuclei of prospective degenerating cells in the posterior portion of the vesiculated region still failed to show silver grains, indicating lack of incorporation

of radioactive precursor; however, silver grains were apparent over nuclei of cells occupying a similar relative position in the anterior portion of the vesiculated region.

Embryos treated with 20 μCi of ^{3}H-uridine provided material suitable for autoradiographs, and the embryos and tissues showed no deleterious effects. After 2 hours of exposure of embryos to ^{3}H-uridine, most of the silver grains were localized over nuclei of epithelial cells located at the basement membrane of the esophagus, whereas fewer silver grains were observed over the cytoplasmic portion of basally located cells (fig. 8). In embryos up to stage 32 (7½ days), a 4-hour exposure to ^{3}H-urdidine showed intense accumulation of silver grains over both nuclei and cytoplasm of laterally disposed epithelial cells. Beyond stage 33 (7½–8 days), there was uniformly intense distribution of silver grains over the esophageal epithelium. Silver grains were found over prospective degenerating cells in both the anterior and posterior portions of the vesiculated esophagus from stage 28 through stage 35 (5½–9 days).

DISCUSSION

The occluded anterior region can be regarded as an area of active DNA synthesis by virtue of the intense incorporation of ^{3}H-thymidine into epithelial cells of that region. Throughout all stages and all treatment times studied, silver grains appeared first over nuclei situated near the basement membrane of the epithelium and later over nuclei in the medial portion of the organ. The epithelial cells located at the basement membrane represent the germinal cells which, after division, yield daughter cells, some of which may occupy an interior position in the occluded esophagus. The resultant daughter cells contain diluted radioactive label. The inability to detect numerous mitotic figures in the occluded region may be due to the very short time interval of the mitotic cycle during which chromosomes are visible. A less likely possibility is that some epithelial cells within the occluded region, having undergone DNA synthesis, delay completion of the mitotic cycle until vesicles appear during the reopening process, since mitotic figures in esophageal cells were frequently distinguished immediately surrounding the vesicles.

Proliferating esophageal epithelial cells in the vesiculated region contain nuclei which undergo interkinetic migration, as evidenced by the incorporation pattern of ^{3}H-thymidine. Autoradiographs of esophageal tissue treated for 1 hour indicate that DNA synthesis occurs only in nuclei located near the basement membrane. When the duration of exposure to ^{3}H-thymidine was increased to 4 hours, radioactivity was observed in nuclei and chromosomes of mitotic figures subjacent to the surfaces of the vesicles, as well as in nuclei located near the epithelial basement membrane. Because a shift in position of ^{3}H-thymidine label from the basement membrane was observed within a 4-hour labeling period, we interpret this as evidence for the interkinetic migration of the epithelial cell nucleus to a position beneath the free surface of the cell. With the nucleus in this position, the proliferating cell enters into metaphase and completes the remainder of the mitotic cycle. Repeating interkinetic migrations of nuclei in esophageal epithelial cells are obscured because of the intense accumulation of silver grains over cells when embryos are exposed to ^{3}H-thymidine for periods exceeding 4 hours.

The interkinetic migration pattern in embryonic esophageal epithelium, particularly in regions containing vesicles, is similar, in many respects, to the pattern observed in the chick embryo neural tube (Sauer and Walker, 1959). The true "germinal zone" in esophageal epithelium is near the basement membrane, while the source of replicating nuclei in the neural tube lies in the mantle zone. However, in both types of epithelium, the nuclei which incorporate ^{3}H-thymidine migrate to a position subjacent to the free surface of the cell to complete the mitotic cycle. In the neural tube, radioactively labeled mitotic figures are conspicuous at

the surface of the neural canal; in the embryonic esophagus, the radioactively labeled mitotic figures are evident at the surface of the vesicle. The time interval required to observe ^{3}H-thymidine-labeled chromosomes at the surface of the neural canal was 4 hours (Sauer and Walker, 1959), which is the same time interval required for radioactively labeled mitotic chromosomes to appear at the surface of a vesicle in the vesiculated portion of the embryonic esophagus.

The failure of select epithelial cells in the developing esophagus to incorporate ^{3}H-thymidine suggests a cessation of DNA synthesis prior to onset of the degeneration process. Similarly, a decrease in incorporation of ^{3}H-thymidine has been suspected in prospectively necrotic cells of the embryonic chick wing bud during the period when certain mesenchymal cells become irreversibly committed to death (Held and Saunders, 1965). Certain esophageal epithelial cells, identified also by position and stage, are prospective degenerating cells, since the selective degeneration of such cells at the appropriate time (stages 34 and 35, *i.e.*, 8–9 days of incubation) permits the reopening of the esophagus (Allenspach, 1966). A gradient of decreasing incorporation of ^{3}H-thymidine within the prospective degenerating cells is suggested by the complete lack or reduced number of silver grains in cells at the extreme posterior end of the vesiculated region of stage 34 and 35 embryos, while, at the same time, cells in relatively similar positions at more anterior levels of the esophagus show incorporation of ^{3}H-thymidine over an 8-hour period of exposure.

Efforts to pinpoint the last DNA replication before necrosis were only partially successful, because of the heterogeneity created by a gradient within the esophageal epithelium. One inherent difficulty which precludes precise correlation of light-microscopic morphology and DNA synthesis in prospective degenerating cells is the lack of identifiable morphological characteristics within the cells. To further confound such studies is the fact that cell death is a very personal thing. We know nothing of the influence of a necrotic cell on adjacent vital cells in esophageal epithelium. Nevertheless, overtly moribund cells appear in a reproducible posterior-to-anterior gradient, and the failure of prospective degenerating cells to incorporate ^{3}H-thymidine suggests that an internal "timing mechanism" (Saunders, Gasseling, and Saunders, 1962) is activated, committing individual cells irreversibly to death. Controls governing the cessation of DNA synthesis in prospective degenerating cells remain highly specific and elusive events.

The embryonic chick esophagus displays a pattern of ^{3}H-uridine incorporation similar to that in the embryonic mouse esophagus (Soriano, 1968). Tritiated-uridine accumulated in nuclei of mouse esophageal epithelial cells after a 30-minute pulse-chase, *in vitro*, and subsequently moved to the cytoplasm (Soriano, 1968). Although pulse-chase experiments were not employed in this study, ^{3}H-uridine accumulated first in epithelial nuclei during 2 hours of exposure to labeled precursor and spread to the cytoplasm within a 4-hour labeling period. The pattern of incorporation of ^{3}H-uridine correlates closely with the cytochemical localization of RNA (Allenspach and Hamilton, 1962). Autoradiographs of material treated with ^{3}H-uridine for 2 hours show a differential incorporation into epithelial cells which are known to be intensely basophilic due to substances that are ribonuclease-sensitive (Allenspach, 1964). This population of cells probably represents the dark cells reported to contain abundant free ribosomes, scattered rough endoplasmic reticulum (Hinsch, 1967; Allenspach, 1969), and a considerable amount of acid phosphatase (Allenspach, 1970). Uridine-incorporation studies failed to reveal a gradient which correlates with the degeneration gradient.

SUMMARY

Autoradiographs of chick embryos (5½–9 days), treated with either ^{3}H-thymidine or ^{3}H-uridine, were employed to examine the pattern of incorporation into the presumptive stratified squamous epithelial cells, prospective degenerating

cells, and mitotic cells of the developing esophagus. Epithelial cells of the occluded region actively participate in cell proliferation during development, with the true germinal cell population localized at the basement membrane. The pattern of incorporation of ^{3}H-thymidine in the vesiculated region suggests that esophageal nuclei undergo interkinetic migration, synthesizing DNA at the basement membrane and moving to the free surface to complete the mitotic phase of the cell cycle. The shortest interval between administration of ^{3}H-thymidine and the appearance of label over mitotic chromosomes was 4 hours. The pattern of incorporation of ^{3}H-thymidine into prospective degenerating cells of the vesiculated region shows that a reduction in DNA synthesis corresponds to the posterior-to-anterior degeneration gradient.

The pattern of ^{3}H-uridine incorporation showed label accumulating in basal cells at the lateral surface of the esophagus in both occluded and vesiculated regions from stage 28 to stage 32 (5½ to 7 days). By stage 33 (7½–8 days), a regionally specific pattern was no longer obvious and all epithelial cells showed intense incorporation. Incorporated ^{3}H-uridine was primarily nuclear after 2 hours, but much label appeared in the cytoplasm by 4 hours of exposure. Some incorporation of ^{3}H-uridine into prospective degenerating cells was observed from stage 28 through stage 35 (5½ to 9 days).

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